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TECHNICAL MANUSCRIPT 626

AN IMPROVED HEAT-STABLE GLUTAMINE-FREE CHEMICALLY DEFINED MEDIUM FOR GROWTH OF MAMMALIAN CELLS

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AUGUST 1970

DEPARTMENT OF THE ARMY
Fort Detrick
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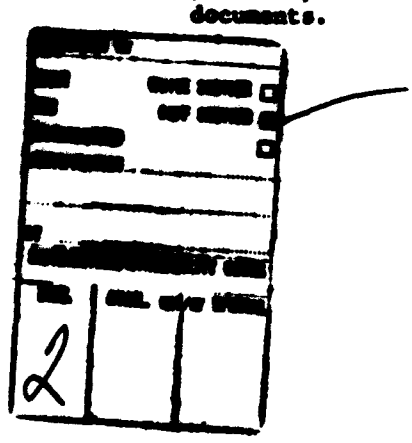

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AN IMPROVED HEAT-STABLE GLUTAMINE-FREE CHEMICALLY DEFINED MEDIUM FOR GROWTH OF MAMMALIAN CELLS

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ABSTRACT

A heat-stable chemically defined medium, free of glutamine, is described for the growth of mammalian cells in suspension culture. The presence of L-alanine in the defined medium permitted the omission of glutamine. A 22-fold increase in the population of a substrain of mouse L cells was obtained (3.4×10^6 cells/ml) in 6 days with no medium replenishment during incubation. Maximum yields (27×10^6 cells/ml) were obtained by daily medium replacement and venting of cultures. Growth was also improved in a line of cat kidney cells and HeLa cells, and in another substrain of L cells.

I. INTRODUCTION*

A heat-stable chemically defined medium reported earlier[1] was improved by increasing the choline level.[2] Glutamine was a necessary component of both media, but it had to be sterilized separately. An autoclavable medium described by Yamane, Matsuya, and Jimbo[3] also required the separate sterilization of glutamine. The present paper describes improvements made to the heat-stable medium that have allowed omission of glutamine and have resulted in higher cell yields.

II. MATERIALS AND METHODS

All ingredients, except methylcellulose and sodium bicarbonate, necessary for a total volume of 1,000 ml of the chemically defined medium (Table 1) were dissolved in 970 ml of hot distilled water. Ten milliliters of 100X vitamin solution were added from storage at -25 C, then 245 ml of the solution were dispensed into 16-oz prescription bottles containing 125 mg of dry methylcellulose. The bottles were autoclaved 15 minutes at 121 C with loosened caps. After cooling, the caps were tightened and the bottles were stored at 5 C. Prior to use, 5 ml of sterile 5% sodium bicarbonate solution were added to each bottle, the caps were tightened to prevent loss of CO_2, and the medium was stored at 5 C. The bicarbonate solution had been sterilized by autoclaving in nearly filled, tightly stoppered 50-ml serum bottles. At time of inoculation, an additional 0.7 ml of bicarbonate solution was added to each 25 ml of medium contained in 100-ml serum bottle culture flasks fitted with rubber stoppers. When the cultures were vented, stoppers were replaced with loose-fitting caps from sterilized 2-oz prescription bottles, and smaller amounts of bicarbonate (depending on cell population) were added to maintain a pH of 7.2 to 7.4. When antibiotics were used in experimental media, sterile solutions (100X) stored at -25 C were thawed and added aseptically to give final concentrations of 100 μg of streptomycin per ml and 100 units of penicillin per ml.

Incubation and cell enumeration procedures were presented previously.[1] Three of the cell lines used, L-SN (a substrain of mouse L-929 cells), HeLa, and cat kidney (CK), were described previously[1] and had been growing in suspension in chemically defined media for 5 to 8 years before the present study. The L-DR cell line (another substrain of mouse L-929 cells), supplied by W.F. Daniels of Fort Detrick, was described by Higuchi[4] and had been growing for approximately 1 year in chemically defined media.

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TABLE 1. HEAT-STABLE, GLUTAMINE-FREE CHEMICALLY DEFINED MEDIUM

Component	Concn, mg/liter	Component	Concn, mg/liter
Amino acids		**Salts, etc.**	
L-Alanine	400	NaCl	7,400
L-Arginine·HCl	100	KCl	400
L-Asparagine	300	$CaCl_2 \cdot 2H_2O$	265
L-Cysteine·HCl	75	Ferric ammonium citrate	3
L-Glutamic acid	150	$MgCl_2 \cdot 6H_2O$	275
L-Histidine·HCl	60	$NaH_2PO_4 \cdot H_2O$	300
L-Isoleucine	150	$ZnSO_4 \cdot 7H_2O$	0.3
L-Leucine	300	Phenol red	10
L-Lysine	300	Methylcellulose, 15 cp	500
L-Methionine	60	$NaHCO_3$	1,000[a]
L-Phenylalanine	120		
L-Proline	300	**Vitamins**[b]	
L-Serine	300	D-Biotin	1
L-Threonine	135	Choline·Cl	50
L-Tryptophan	60	Folic acid	1
L-Tyrosine	120	Niacinamide	1
L-Valine	150	Calcium pantothenate	2
		Pyridoxal·HCl	1
Carbon sources		Thiamine·HCl	1
Glucose	3,000	i-Inositol	1
Sodium pyruvate	110	Riboflavin	0.1
		B_{12}	0.002

a. Added aseptically as 5% solution.
b. Added as a 100X solution during medium preparation.

III. RESULTS

The composition of the medium developed in these studies is given in Table 1. The medium differs from that previously reported[3] by (i) omission of glutamine, (ii) addition of L-alanine, L-asparagine, L-glutamic acid, L-proline, L-serine, iron, and zinc, and (iii) an increase in the concentrations of glucose and $NaH_2PO_4 \cdot H_2O$ from 1,000 mg and 100 mg per liter, respectively.

A comparison of the effectiveness of the medium and the effect of the variation of composition on the growth of L-DR cells is given in Table 2. Alanine and serine were essential for L-DR cells, but evidence for improved cell growth with asparagine and proline was equivocal. Similar results were obtained with L-SN cells with the exception that serine was not essential. Asparagine and proline were included because many experiments with L-SN cells demonstrated that these amino acids reduced the lag phase on transfer of cells from glutamine-containing to glutamine-free media. In the presence of asparagine and proline, a lag in growth of 1 or 2 days was occasionally noted when L-DR cells from medium containing glutamine were inoculated into glutamine-free medium. Glutamic acid was stimulatory in the absence of glutamine. The phosphate and glucose concentrations were increased to insure sufficient levels for higher cell populations. Iron and zinc were added as a result of studies by Thomas and Johnson[5] and Higuchi.[4]

TABLE 2. GROWTH OF L-DR CELLS IN HEAT-STABLE MEDIUM WITH NO MEDIUM REPLENISHMENT

Omission from Medium	Growth,[a] number cells x 10^6/ml		
	5 Days	6 Days	7 Days
None (medium as Table 1)	1.63	2.90	2.76
Alanine	0.37	0.47	0.49
Asparagine	1.42	2.61	2.48
Glutamic acid	0.95	1.52	2.47
Proline	1.38	2.13	2.56
Serine	0.45	0.21	0.12
Ala, asp, glutamate, prol, ser	0.13	0.12	0.12
Fe	1.43	1.91	2.38
Zn	1.36	1.69	2.46
$NaH_2PO_4 \cdot H_2O$ reduced to 150 mg/liter	1.38	2.05	2.43

a. Zero-day count was 0.22 x 10^6/ml.

The data in Table 3 compare the growth of four cell lines without replenishment of medium. Table 4 shows the maximum growth obtained when media were changed daily and cultures were vented after populations reached 2 to 3 million cells per ml. Data previously reported for cells grown in the heat-stable glutamine medium[1] are given for comparison. The failure of L-DR cells to grow in the glutamine-containing medium is explained by the absence of serine in that medium.

TABLE 3. GROWTH OF VARIOUS CELL LINES IN THE GLUTAMINE-FREE HEAT-STABLE MEDIUM WITH NO MEDIUM REPLENISHMENT

Cell	Growth, no. cells x 10^6/ml		Days of Incubation	Fold Increase
	0 Day	Final Growth		
CK	0.21	3.08	7	14.7
HeLa	0.25	1.27	7	5.1
L-DR	0.15	3.36	6	22.4
L-SW	0.19	2.04	6	10.7

TABLE 4. GROWTH OF CELLS IN HEAT-STABLE MEDIUM WITH MEDIUM REPLENISHMENTS

Cell	Maximum Growth, no. cells x 10^6/ml	
	Improved Glutamine-Free Medium	Glutamine Medium[a]
CK	28.5	3.4
HeLa	4.0	3.4
L-DR	27.3	0[b]
L-SW	10.5	4.7

a. Data were reported previously.[1]
b. L-DR cells did not grow in this medium because serine was not present.

Results of testing for the heat stability of the medium are shown in Table 5. Autoclaving for 30 minutes did not impair growth of cells. The L-DR cells were subsequently subcultured several times in media that were autoclaved for 30 minutes and no diminution in growth was noted.

TABLE 5. COMPARISON OF GROWTH OF L-DR CELLS IN FILTERED AND AUTOCLAVED MEDIA

	Growth, no. cells x 10^6/ml		
	No Medium Replenishment, Venting		Medium Changed Daily 5 Through 12 Days and Cultures Vented
Sterilization	0 Day	5 Days	12 Days
Filtration[a]	0.20	1.49	17.4
Autoclaved 15 min[b]	0.18	1.59	20.4
Autoclaved 30 min	0.17	1.25	18.5

a. 0.22-μ membrane filter.
b. At 121 C.

IV. DISCUSSION

The primary intent of this paper was to present the formulation of a heat-stable chemically defined medium that supported increased populations of several cell lines. The heat-stable medium described earlier[1] had been markedly improved by increasing the choline concentration,[2] resulting in considerably higher cell populations. Glutamine and bicarbonate were the heat-sensitive components of the earlier medium[1] that required separate sterilizing procedures; therefore, eliminating one of these components was considered useful. In the present work it was not expected that high cell yields could be obtained in a culture medium completely free of glutamine in view of results of earlier workers.[6] Glutamine has been used in all previous cell culture media, maintaining a status of importance similar to that occupied for many years by serum as a basic medium constituent. For example, the apparent necessity for glutamine and serum for cells is emphasized by their inclusion as aseptic additives (with sodium bicarbonate) even in a medium that is otherwise autoclavable.[3]

In addition to our primary objective, i.e., the formulation of an improved heat-stable medium, some very interesting nutritional interactions were noted. They may be summarized as follows: (i) for L cells,

L-alanine was essential in the absence of L-glutamine; (ii) L-glutamic acid did not replace L-glutamine in the growth of L cells; (iii) L-serine was essential for growth of an L cell substrain (L-DR).

Eagle et al.[6] had shown that glutamine was a requirement for both HeLa and L cells, but that a mixture of six nonessential amino acids had a sparing effect on this requirement. They found also that glutamic acid would substitute for glutamine in the growth of HeLa cells but not for L cells. In a later paper, Eagle et al.[7] reported that HeLa, Chang's conjunctiva, and strain T diploid human fibroblast cells that had been adapted to grow in high glutamic acid medium were able to grow in a medium lacking both glutamic acid and glutamine if high cell concentrations were used as inocula. By contrast, monkey kidney cells, having a high glutamine synthetase activity, did not require adaptation to growth with glutamic acid. Griffiths and Pirt,[8] in studies on amino acid utilization during growth of animal cells, also adapted cells to grow in media containing reduced levels of glutamine with high levels of glutamic acid. The above three groups of workers, in contrast to our work, used serum or serum proteins as essential medium components.

In our studies with a chemically defined system, glutamic acid was inhibitory at concentrations above 2 mM. Subsequent studies showed that in the absence of glutamine but in the presence of alanine, growth of L-DR cells was stimulated by asparagine, proline, and 1 mM glutamic acid. The essentiality of alanine for growth of animal cells has not been described previously to our knowledge, but serine has been reported as a requirement for the growth of a HeLa cell line and for subsequent replication of Newcastle disease virus.[9]

The problem of the possible toxicity of excessive levels of ammonium ions produced in the medium by cells as a result of glutamine metabolism has been discussed by Higuchi.[4] Griffiths and Pirt[8] and others have also shown that enzymic and nonenzymic glutamine decomposition may be a problem in the development of media that are able to support large cell population densities. The absence of glutamine in our medium obviates these difficulties.

Sodium bicarbonate remains as a necessary aseptic additive to the autoclaved medium. Although it has been shown that hypoxanthine plus orotic acid or other combinations of purines and pyrimidines would partially substitute for $NaHCO_3$[10] and that 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid was an efficient buffer in the absence of $NaHCO_3$,[11] neither of these modifications permitted satisfactory growth in the present medium. Future studies may resolve this problem.

The chemically defined, heat-stable, glutamine-free medium described above, capable of producing populations of L cells in suspension culture exceeding 25×10^6/ml, should prove very useful in future cell culture studies and have broad applicability in the areas of routine cell maintenance, growth studies, and virus research.

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13. ABSTRACT

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